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(54) Title: HUMAN INTERLEUKIN-8 ANALOGS

(57) Abstract

Human interleukin-8 (IL-8) analogs that are modified in the Glu4Leu5Arg6 region, and have a core structure corresponding to the IL-8(7-51) sequence are provided. These neutrophil binding analogs display altered IL-8 activities that can be exploited for therapeutic and other purposes. Such antagonists include those in which, for example, the Leu[5] and/or Arg[6] residues are replaced, and in which the Glu[4] and/or Leu[5] residues are deleted. Also provided are biologically active human interleukin-8 (IL-8) analogs comprising a core sequence that includes IL-8(1-51), IL-8(3-51) or IL-8(4-51). This invention also provides pharmaceutical compositions containing the aforementioned analogs.

HUMAN INTERLEUKIN-8 ANALOGS

TECHNICAL FIELD

This invention relates to the human cytokine, interleukin-8.

5   BACKGROUND OF THE INVENTION

A human cytokine that promotes the recruitment and activation of neutrophil leukocytes has been identified as one of several endogenous mediators of the acute inflammatory response. In the past it was variously termed
10   neutrophil-activating factor, monocyte-derived neutrophil chemotactic factor, interleukin-8 (IL-8), and neutrophil-activating peptide-1. IL-8 appears to have gained the widest acceptance and the term will be used herein.

The most abundant naturally occurring form of the IL-8
15   monomer is a 72-residue protein apparently derived by processing of a 99-residue precursor. Other proteins with related sequences, including neutrophil-activating peptide-2 and GROα (with melanoma growth stimulatory activity) are IL-8 homologues which have neutrophil-activating
20   properties.

The in vitro effects of IL-8 on neutrophils are similar to those of other chemotactic agonists such as C5a and fMet-Leu-Phe and include induction of a transient rise in cytosolic free calcium, the release of granules
25   containing degradative enzymes such as elastase, the respiratory H_2O_2 burst, neutrophil shape change, and chemotaxis. IL-8 appears to bind to at least one class of receptor sites on neutrophils with a frequency of approximately 64,000/cell and a K_d of 0.2 nM.

30   The three-dimensional structure of IL-8 is known by two-dimensional NMR and x-ray diffraction techniques. The IL-8 monomer has antiparallel β strands followed by a single overlying COOH-terminal α helix. Two disulfide bridges, between cysteines 7 and 34, and between cysteines

-2-

9 and 50 seem to stabilize the tertiary structure.
Residues 1-6 and the loop residues 7-18 seem to have little
defined secondary structure. In solution, IL-8 is a
noncovalent homodimer which is stabilized primarily by
5 interactions between the ß strands of the two monomers.

Examination of the three-dimensional structure
indicates that following the cysteine at position 50, the
residues form a type 1 ß turn (at residues 51 to 55)
followed by an amphipathic α helix (at residues 55 to 72)
10 that transverses the ß sheet. The hydrophobic face of the
α helix interacts with and stabilizes the hydrophobic face
of the ß sheet. Some of the interactions are between the
two subunits of the dimeric molecule.

As it is established that IL-8 is a key mediator of
15 inflammatory diseases, it would be desirable to identify
substances capable of blocking or interrupting the activity
of IL-8 for use in anti-inflammatory compositions. Such
compositions may prove to be advantageous over presently
available steroid based anti-inflammatory drugs which often
20 have severe side-effects with the continued usage that is
required for chronic inflammatory diseases. It would also
be desirable to identify IL-8 analogs having an increased
inflammatory activity for medical research applications.

The investigation described herein arose as a result
25 of an investigation of the functioning of the IL-8 cytokine
carried out by production of structural analogs of IL-8.

IL-8 has been previously produced through chemical
synthesis (for example see: Clark-Lewis, et al "Chemical
Synthesis, Purification, and Characterization of Two
30 Inflammatory Proteins; Neutrophil-Activating Peptide-1
(Interleukin-8) and Neutrophil-Activating Peptide-2" (1991)
Biochemistry 30: 3128-3135) and by recombinant DNA methods
(for example see: Hebert, et al "Scanning Mutagenesis of

-3-

Interleukin-8 Identifies A Cluster of Residues Required for Receptor Binding" (1991) J . Biol. Chem. 286: 18989-18994). Such methods of synthesis make it possible to produce analogs of IL-8 in order to investigate such aspects of the cytokine as the receptor binding site(s). In addition, it is known that IL-8 exists in several forms that vary at the NH_2 -terminus, which have been detected in preparations purified form natural sources. These variations correspond to the predominant 72-residue form (which is generally considered to be the prototype IL-8 molecule); a 77-residue form having 5 additional NH_2 -terminus amino acids on each monomer; and, two shortened forms having residues 3-72 and 4-72 of the 72 amino acid form, respectively.

SUMMARY OF THE INVENTION

The inventors herein have investigated several structural analogs of IL-8 and have discovered that manipulation of the 72-residue form of IL-8, particularly in the N-terminal region thereof, yields IL-8 analogs having therapeutically useful properties. More particularly, the IL-8 analogs of the present invention comprise an amino acid sequence substantially equivalent to the IL-8 sequence beginning at residue 4 and continuing at least to residue 51, wherein at least one of the N-terminal residues found to be critical for neutrophil binding and stimulation, i.e., Glu^4-Leu^5-Arg^6, is either replaced or deleted.

In embodiments of the invention, the Glu^4-Leu^5-Arg^6 region of IL-8 is modified selectively to provide antagonists of IL-8. In one particular embodiment, antagonists that compete strongly with IL-8 for neutrophil binding are obtained by replacing or deleting at least residue Leu^5. In another particular embodiment, antagonists that compete only weakly with IL-8 yet still retain antagonist properties are obtained by replacing at least

-4-

residue Arg[6].

This invention also provides pharmaceutical compositions of the aforementioned analogs, comprising the analog and a suitable carrier therefor. Also provided are
5   methods of the use of the aforementioned analogs.

IL-8 analogs having the first 2 or 3 residues at the NH$_2$ -terminus of the 72-residue monomer deleted so as to provide the 3-72 and 4-72 forms are useful as enhanced inflammatory mediators. It has now been found that C-
10 terminally truncated analogs of IL-8(3-72) and IL-8(4-72) have significant biological activity.

Accordingly, this invention also provides a biologically active human interleukin-8 (IL-8) analog having an amino acid sequence substantially equivalent to
15 the IL-8 1-72 sequence beginning at residue 4 and continuing to a COOH-terminus at residue 51 or a residue between residue 51 and residue 72. This invention also provide the preceding analog that additionally comprises residue 3 of IL-8 1-72 or additionally comprises residues
20 1-3 of IL-8 1-72.

This invention also provides pharmaceutical compositions comprising the aforementioned biologically active analogs together with a suitable carrier therefor.

The invention also provides methods of use of the
25 aforementioned analogs. In addition, this invention also provides methods of use of IL-8 3-72 and IL-8 4-72 to activate human neutrophils and pharmaceutical compositions suitable therefor.

DESCRIPTION OF THE DRAWINGS
30   For better understanding of the invention, reference

-5-

may be made to the preferred embodiments and examples described below, and the accompanying drawings, in which:

5

Figure 1 is a graph showing neutrophil elastase release activity of NH-terminal deletion IL-8 analogs. Shown are determinations at the indicated concentrations of IL-8 1-72: ●, 1-72; ▲, 3-72; ▵, 4-72; ◊, 5-72; ■, 6-72; □, 7-72; and ○, 77-residue IL-8. Unidirectional error bars indicate the standard deviations. Data are representative of three assays using different neutrophil preparations.

10

Figure 2 is a graph showing neutrophil chemotaxis activity of NH_2-terminal deletion IL-8 analogs. The chemotaxis index (stimulated migration/control random migration) was determined at the indicated concentrations of IL-8 1-72: ●, 1-72; ▲, 3-72; ▵, 4-72; ◊, 5-72; ■, 6-72; □, 7-72; and ○, 77-residue IL-8. Unidirectional error bars indicate the standard deviations. Data are representative of three assays using different neutrophil preparations.

15

20

Figure 3 is a graph showing neutrophil elastase release activity of COOH-terminal deletion IL-8 analogs. Shown are determinations at the indicated concentrations of IL-8 1-72: ●, 1-72; ○, 1-69; ▲, 1-66; ▵, 1-63; ■, 1-60; □, 1-58; ◊, 1-54; and X, 1-51. Unidirectional error bars indicate the standard deviations. Data are representative of three assays using different neutrophil preparations.

25

Figure 4 is a graph showing neutrophil chemotaxis activity of the COOH-terminal deletion IL-8 analogs. The chemotactic index (stimulated migrations/control random migration) was determined at the indicated concentrations of IL-8 1-72: ●, 1-72; ○, 1-69; ▲, 1-66; ▵, 1-63; ■, 1-60; □, 1-58; ◊, 1-54; and X, 1-51.

30

-6-

Unidirectional error bars indicate the standard
deviations. Data are representative of three assays
using different neutrophil preparations.

Figure 5 is a graph showing competitive binding of the
NH$_2$-terminal deletion IL-8 analogs to neutrophils. The
percentage of specific ^{125}I-labelled IL-8 1-72 counts
competed from purified neutrophils were calculated
after subtraction of nonspecific binding. Shown are
determinations at the indicated concentrations of IL-8.
1-72: ●, 1-72; ▲, 3-72; △, 4-72; ◊, 5-72; ■, 6-72; □,
7-72; and o, 77-residue IL-8.

Figure 6 is a graph showing competitive binding of the
COOH-terminal deletion IL-8 analogs to neutrophils.
The percentage of specific ^{125}I-labelled IL-8 1-72
counts competed from purified neutrophils were
calculated after subtraction of nonspecific binding.
Shown are determinations at the indicated
concentrations of IL-8 1-72: ●, 1-72; o, 1-69; ▲, 1-
66; △, 1-63; ■, 1-60; □, 1-58; ◊, 1-54; and X, 1-51.

Figure 7 is a graph showing neutrophil elastase
release activity and synergistic activity of NH- and
COOH-terminal IL-8 peptides. Shown are determinations
at the indicated concentrations of the NH-terminal
peptide corresponding to residues 1-10 of 77-residue
IL-8 (■), the COOH-terminal peptide corresponding to
residues 51-72 of IL-8 (□), and the indicated
concentrations of the NH$_2$ -terminal peptide with 10^{-7}
M IL-8 6-72(o), and the COOH-terminal peptide with 10^{-7}
M of IL-8 1-51 (●).

Figure 8 is a graph showing elastase release by human
neutrophils stimulated with IL-8 1-72 in the presence
of increasing concentrations of IL-8 5-72 (▲), IL-8 6-
72 (●) and IL-8 7-72 (■).

-7-

Figure 9 is a graph showing the results of a competitive binding study with neutrophils incubated with labelled IL-8 1-72 in the presence of increasing concentrations of unlabelled IL-8 1-72 (○), IL-8 5-72 (◊), IL-8 6-72 (▲), and IL-8 7-72 (□).

Figure 10 is a graph showing elastase release by neutrophils stimulated with IL-8 1-72 (●) and Fmet-Leu-Phe (▲) in the presence of increasing concentrations of IL-8 6-72 and, the effect of IL-8 6-72 alone (○).

Figure 11 is a graph showing neutrophil chemotaxis migration in the presence of IL-8 6-72 and IL-8 1-72 (●) and, in the presence of IL-8 6-72 alone (○).

Figure 12 is a graph showing elastase release by neutrophils stimulated with IL-8 (●); GROα (■) and NAP-2 (▲) in the presence of increasing concentrations of IL-8 6-72.

Figure 13 provides graphs showing elastase release by neutrophils induced with 10nM IL-8(4-72) (Figs 13A and 13C); and competition for ^{125}I-IL-8 binding to human neutrophils. Analogs used were IL-8,AAR(7-72) (■), IL-8,ELQ(7-72) (▲), IL-8,ELL(7-72) (◆), IL-8(6-72) (●) (Figs. 13A and 13B), and IL-8,IR(7-72) (■), and IL-8,QR(7-72)(▲)(Figs. 13C and 13D). Agonistic effects of IL-8,IR(7-72) (□) and IL-8,QR(7-72)(▲) at high concentrations (Fig.13C) as well as the competition by unlabelled IL-8(4-72) (○) are also shown.

Figures 14 and 15 respectively show oedema and PMN accumulation responses in 4 separate experiments with 6 replicates for each injection; mean I s.e.m. for each rabbit. In each case identified by a numeral from 1 - 12, the injections are (1) PBS (vehicle); (2)

-8-

IL-8 1x10^{-11} moles/site; (3) IL-8 1x10^{-11} + AAR7-72 1x10^{-11} moles/site; (4) IL-8 1x10^{-11} + AAR7-72 1x10^{-10} moles/site; (5) IL-8 1x10^{-11} + 6-72 1x10^{-10} moles/site; (6) IL-8 1x10^{-10} moles/site; (7) IL-8 1-10^{-10} + AAR7-72 1x10^{-11} moles/site; (8) IL-8 1x10^{-10} + AAR7-72 1x10^{-10} moles/site; (9) IL-8 1x10^{-10} + IL-8 6-72 1x10^{-10} moles/site; (10) AAR7-72 1x10^{-11} moles/site; (11) AAR7-72 1x10^{-10} moles/site; (12) 6-72 1x10^{-10} moles/site.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The 72-residue form of the IL-8 monomer from the NH$_2$-terminus to the COOH-terminus is as follows:

SEQ ID NO: 1

```
Ser Ala Lys Glu Leu Arg Cys Gln Cys Ile Lys Thr Tyr Ser Lys
              5                  10                  15
Pro Phe His Pro Lys Phe Ile Lys Glu Leu Arg Val Ile Glu Ser
             20                  25                  30
Gly Pro His Cys Ala Asn Thr Glu Ile Ile Val Lys Leu Ser Asp
             35                  40                  45
Gly Arg Glu Leu Cys Leu Asp Pro Lys Glu Asn Trp Val Gln Arg
             50                  55                  60
Val Val Glu Lys Phe Leu Lys Arg Ala Glu Asn Ser
             65                  70
```

Throughout this specification, reference to residue numbers in IL-8 analogs will be a reference to the numbered residues shown above. For example, when the first 2, 3, 4 or 5 residues at the NH$_2$-terminus are deleted, the analogs will be referred to as the 3-72, 4-72, 5-72, and 6-72, forms respectively. Replacement amino acids in the IL-8 analogs will be referred to by their identity and location within the sequence shown above. Thus, an analog of IL-8(4-72) in which the Arg residue at position 6 is replaced with Ile, for instance, will be referred to as

Glu⁴Leu⁵Ile⁶(7-72) or, more simply by single letter code, as
ELI(7-72). Such forms may be prefixed with the term IL-8 as
a complete denomination. Other analogs will be referred to
by similar notations with reference to the above 72-residue
5   sequence.

With reference to the sequence shown above, IL-8
analogs of the present invention may comprise an amino acid
sequence that is either identical to the illustrated
sequence, or to a region thereof as herein defined, or may
10 comprise an amino acid sequence that is "substantially
equivalent" thereto. Sequences that are "substantially
equivalent" are characterized by from 1 to 10, e.g. up to
5, amino acid deletions or replacements that do not cause
a statistically significant change in the activity of the
15 analog, relative to a counterpart comprising an amino acid
sequence identical to the sequences shown above. Whether
a change in activity resulting from an amino acid change or
deletion is statistically significant will of course be
determined in the context of the assay used to identify the
20 given activity.   Known three-dimensional modelling
techniques may be used to design and construct further
analogs of this invention wherein conservative alterations
are made within the core sequences described above which
will not prevent the analog from binding to the neutrophil.
25 It is expected that many such conservative alterations may
be made, particularly in the C-terminal region beyond
residue 35. In making such modifications it is expected
that it will be necessary to retain the characteristic
disulfide bridges of the IL-8 monomer. For example,
30 replacement of Cys 9 and Cys 50, or Cys 7 and Cys 34 with
amino butyric acid (ABA) in IL-8, results in a loss of
elastase release activity.

Specific amino acid replacements that may be tolerated
include: Lys 15→Arg; Tyr 13→Phe; Ile 10→Val; His 33→Ala;
35 His 33→Glu; His 33→Gln; His 33→Ser; Ser 14→Thr; Thr 12→Ser;

-10-

Lys 11→Arg; Gln 8→Leu; Arg 26→Glu; Lys 23→Glu; Glu 29→Lys, since these substitutions in IL-8 do not significantly affect elastase release activity.

5   The IL-8 analogs of this invention may be synthesized chemically by a variety of known means or according to the specific examples herein. The analogs may also be synthesized by a variety of known recombinant DNA techniques such as those described by Hebert, et al (1991) [supra]; Hebert, et al (1990) J. Immunol. 145: 3022-3040; 10 or, Lindley, et al (1988) Proc. Natl. Acad. Sci. U.S.A. 85: 9199-9203. To produce the analogs described herein it is only necessary to delete or replace the codons in the nucleic acid sequences employed that correspond to the amino acid residues that are to be replaced or deleted.

15   Cytokine analogs of IL-8 are formed by permitting the analog monomers to fold and associate in the dimer form. Folding is accomplished by permitting the formations of the disulfide bridges by oxidation of the appropriate half cysteines, for example by the procedures described in 20 Clark-Lewis, et al (1988) Proc. Natl. Acad. Sci. U.S.A. 85: 7897-7902; Woo, et al (1989) Protein Eng. 3: 39-37; and Clark-Lewis, et al (1991) [supra]. The monomers will spontaneously associate by hydrogen bonding in solution.

   As will be described below in further detail, IL-8 25 biological activity is retained when certain COOH-terminal residues are deleted but potency is progressively reduced as the COOH-terminal residues are excluded. Some activity is retained in a 1-51 residue analog wherein the entire COOH-terminal α and β turns are missing. For biological 30 activity, it is preferred that the deletions be only of the COOH-terminal residues in the region 67-72 as a significant amount of biological activity is shown to be retained by the IL-8 1-66 analog and enhanced activities are demonstrated in the 1-69 analog. In the preferred analogs

-11-

which provide enhanced neutrophil activation, the NH$_2$ - terminus will be either the 3rd or 4th residue and the sequence of the monomer will continue to a desired COOH-terminus, preferably in the area of residues 66-72. Analog 1-69 also has potent biological activities.

For IL-8 mediators with low neutrophil activation activity and strong chemotaxis activity, the preferred analogs are derived from the 5-51 form as a core sequence, preferably with further COOH-terminus residues being present, and most preferably with the COOH-terminus being residue 66-72.

In a valuable aspect of the present invention, there are provided IL-8 analogs that are antagonists of IL-8 activity. Antagonists of IL-8 activity are characterized by the ability to reduce one or more of the measurable consequences of IL-8-mediated neutrophil stimulation. Biological assays are known which may be used to test for IL-8 activities or an ability to block or inhibit IL-8. The in vitro assays require human neutrophils which may be isolated from donor blood by known procedures such as that described by Peveri, et al (1988) J. Exp. Med. 167: 1547-1559. The assays include measurement of elastase release, cytosolic free calcium changes, and chemotaxis and may be performed according to methods described by Schroder, et al (1987) J. Immunol. 139: 3474-3483; and, Peveri, et al (1988) [supra].

Analogs of IL-8 having IL-8 antagonist activity may be further characterized by their ability to compete with IL-8 for neutrophil binding. Analogs that compete relatively strongly exhibit a Kd value that is less that about 50nM, as determined using the assays herein described. According to embodiments of the invention, IL-8 analogs that have IL-8 antagonist activity and compete relatively strongly with IL-8 for neutrophil binding comprise an amino acid sequence

-12-

substantially equivalent to IL-8(4-72), wherein the Glu^4-Leu^5-Arg^6 region is modified to confer on the analog a Kd value of less than about 50nM. Modifications suitable for this purpose include deletion of Glu^4 and replacement of
5 Glu^4 and/or Leu^5. Replacement amino acids may be selected from among any of the naturally occurring and synthetic amino acids, including but not limited to amino acids having a hydrophobic side chain. Specific replacement amino acids include alanine, isoleucine, glutamine and
10 leucine. Preferred embodiments of the present invention include the following human IL-8 analogs: $Ala^4Ala^5(6-72)$; $Ile^5(6-72)$; $Gln^5(6-72)$; and (6-72).

Analogs that compete relatively weakly with IL-8 for neutrophil binding yet exhibit relatively strong
15 antagonistic activity, as determined in the elastase release assay, are also provided by the present invention. This remarkable combination of characteristics has surprisingly also been found in IL-8 analogs comprising an amino acid sequence substantially equivalent to IL-8(4-72),
20 wherein the Glu^4-Leu^5-Arg^6 region is modified to confer on the analog a Kd value of greater than about 50nM. Modifications capable of yielding analogs of this type include replacement of the Arg^6 residue. Replacement amino acids may be selected from the naturally occurring and
25 synthetic amino acids, including but not limited to those having a hydrophobic side chain. Specific replacement amino acids include leucine, norleucine (Nle) and lysine. Preferred embodiments of the present invention include the following human IL-8 analogs: $Glu^4Leu^5Leu^6(7-72)$;
30 $Glu^4Leu^5Nle^6(7-72)$; and $Glu^4Leu^5Lys^6(7-72)$.

For IL-8 antagonists, preferred analogs are derived from at least the 6-51 form as a core sequence with further COOH-terminus residues being optionally present, up to and including residue 72.

-13-

Methods of in vitro use of the analogs of this
invention either to bring about neutrophil activation or to
block the effects of IL-8 or similar compounds on
neutrophils will be readily apparent from the examples
5   herein. In addition, methods of in vivo use, particularly
in humans, will be readily apparent to those skilled in the
art. To limit inflammation in humans, one or more of the
antagonists may be administered, for example through
intravenous   injection,   inhalation,   or   by   oral
10   administration, particularly when formulated with a
suitable carrier in a pharmaceutical composition. The
analogs which have IL-8 activity may be used to activate
neutrophils. For example, cytokines of such analogs may be
administered to an animal, particularly a human, in order
15   to stimulate an inflammatory response. The analogs that
bring about a strong chemotaxis activity such as the 5-72
form may be administered to attract neutrophils to an area
of disease. For those cytokine analogs having a greater
potency than IL-8 such as IL-8 3-72 and IL-8 4-72,
20   inflammation response or neutrophil activation may be
enhanced.

A combination of analogs or analogs and other,peptides
may be used. For example, the 1-51 analogs may be employed
for its ability to bind to neutrophils but cause a low
25   level of neutrophil activation; subsequently, the 51-72
peptide may then be used or administered to boost the
neutrophil activating activity of the 1-51 analog.

The analog monomers of the invention may be
synthesized according to the following protocol. A fully
30   automated peptide synthesizer (Applied Systems 430A) is
used. The synthesis is started with a protected C-terminal
amino acid linked to a cross-linked polystyrene resin via
a 4-(carboxamidomethyl)benzyl ester linkage (the so-called
pam resin) (0.4 mmol of 0.8 mmol/g of aminoacyl resin). N^α-
35   t-Boc acids with appropriate side chain protecting groups

-14-

are added in a stepwise fashion until the entire protected polypeptide chanin is formed. Side chain protection is as follows: benzyl (Asp, Gly, Ser, and Thr); 4-methylbenzyl (Cys); toluenesulfenyl (Arg); 2-chlorobenzyloxycarbonyl (Lys); 2-bromobenyloxy-carbonyl (Tyr); formyl (Trp); dinitrophenyl (His); and none (Ala, Asn, Gly, Gln, Ile, Leu, Met, Phe, Pro, Val). Samples may be taken after each step to retrospectively monitor the amino acid coupling yields using a ninhydrin-based reaction following the procedures of Sarin, et al (1981) Anal. Biochem. 117: 147-157. The protected polypeptide resin is treated twice for 30 min with 2-mercaptoethanol (20%) in dimethylformamide containing diisopropylethylamine (5%) to remove the DNP groups form the histidine side chains. The resin is dried and cleaved using the "low-high" hydrogen fluoride method as described by Tam, et al (1983) J. Am. Chem. Soc. 105: 6442-6485 except for the following modifications. After the 25% hydrogen fluoride step, the partially protected peptide resin is filtered from the reaction mixture by using an all-Teflon filtration apparatus fitted with a Zitex filter and washed with dichloromethane and dried before the high 90% hydrogen fluoride step. The ethyl acetate precipitate of the material released form the resin is dissolved in 50 ml of 6 M guanidine hydrochloride, 0.1 M Tris-acetate, pH 8.5, and 20% 2-mercaptoethanol and stirred at 37°C for 2 h and then acidified with 2 mil of acetic acid. This mixture is the crude peptide product.

Alternately, histidine may be protected with π benzyloxymethyl instead of dinitrophenyl. The π benzyloxymethyl group is acid labile thus eliminating the need for thiolysis of the dinitrophenyl group before and after hydrogen fluoride deprotection. Acetylation is carried out on the N^a deprotected but otherwise fully protected peptide resin using acetic anhydride (10%) in dimethyl formamide.

-16-

The crude peptide product may be purified and folded
according to the following protocol. Three different C-18
silica HPLC columns may be used in the purification and
analysis of the peptide, including a preparative column
5   (22.4 x 250 mm column with at 22.4 x 100 mm guard column)
packed with 12 μm, 300-A pore size packing (Dynamax, Rainin
Instrument Co., Woburn, MA.); a semipreparative column (10
x 250 mm) Vydac C-18 column, with 5-μm particle, 300-A
pore-size packing (Separations Group, Hesperia, CA); and an
10   analytical column (4.6 x 250 mm) (Vydac) containing the
same packing. The crude peptide product is loaded onto the
preparative column and the retained material eluted with a
0-60% water-acetonitrile gradient in 0.1% trifluoracetic
acid over 4 h at a flow rate of 15 ml/min. A sample (25
15   μl) of fractions containing 225-nm UV-absorbing material
are rerun on the analytical column using reverse phase HPLC
with the above described elution gradient run over 1 h. By
comparison   with   the   profile   of   the   crude   material,
fractions   containing   the   major   peak   are   pooled   and
20   lyophilized.   This   material   is   reconstituted   in   1   M
guanidine hydrochloride and Tris-acetate, pH 8.5, at a
concentration of 0.2 mg/ml and stirred vigorously overnight
in an open beaker so the air was kept bubbling through the
mixture   by   vortex   action.   This   procedure   promotes
25   formation   of   the   disulfide   bridges   by   oxidation   of   the
appropriate half-cysteines. The material is acidified with
2 ml   of   acetic   acid,   and   half   was   loaded   onto   the
semipreparative   column   and   the   retained   material   eluted
with the same gradient as described above at a flow rate of
30   3 ml/min.   Samples   of   each   fraction   are   run   on   the
analytical   column.   Fractions   containing   only   material
with the retention time of the major peak in the folded
material are pooled and lyophilized.

An assay for free sylfhydryls using Ellman reagents,
35   as described by Clark-Lewis et al (1988) Proc. Natl. Acad.
Sci. U.S.A. 65: 7897-7902, may be used to determine the

-16-

extent of folding. In addition, folding may be monitored
on the analytical HPLC column by observing the appearance
of a peak corresponding to the folded form that has a
retention time approximately 3 min. earlier than the
5 reduced form. In the examples below, at least 80% folding
was observed except in respect of the 7-72 analog which,
when acetylated at the NH-terminus, was found to fold
quantitatively. All the purified folded analogs in the
following examples failed to react with Ellman's reagent,
10 indicating the absence of free cysteine.

Analog purity may be assessed on the analytical HPLC
column or by other means such as isoelectric focusing. A
protocol for isoelectric focusing is as follows. Mini
polyacrylamide gels (Pharmacia "PHAST" gels, IEF 3-9;
15 Pharmacia, Uppsala, Sweden) are washed in 8 M urea and then
in 8 M urea containing pH 9-11 Ampholytes (Pharmacia), for
30 min each, either with or without 10 μg/ml
dithiothreitol. Gels are prerun for 15 V-h at 200-V, 2.0-
mA, 3.0-mW maximum settings, and the samples are loaded and
20 run for 410 v-h at 1000-V, 5.0-mA, 3.0-mW maximum settings
on the Pharmacia "PHAST" system for a total of 500-V with
maximum settings of 2.0-mW, 5.0-mA, and 100-V. The pH
gradient may be determined by using a surface pH electrode.
The gels are stained with silver by using the "PHAST"
25 developing system as described in the manual.

The structure of the analogs may be determined by
protein sequencing, for example by using the following
protocol. Protein sequences are determined by Edman
degradations using either solid-phase or gas-liquid-phase
30 methods. For solid-phase sequence analysis, reduced and
carboxymethylatedprotein or proteolytic cleavage fragments
are   coupled   to   arylamine-functionalized
poly(vinylidenedifluoride) membranes (Sequelon AA;
Milligen/Biosearch, Burlington, MA) using the water-
35 s o l u a b l e   c a r b o d i i m i d e   1 - e t h y l - 3 - [3 -

-17-

(dimethylamino)propul]carbodiimide   hydrochloride   and
sequenced in a Milligen/Biosearch Model 6600 sequencer
using standard protocols. For gas-liquid-phase sequence
analysis, polypeptides are applied to Polybrene-coated
5 glass fibre disks and sequenced in an Applied Biosystems
Model 477 protein sequencer using standard protocols.
Sequencing of protected peptide resins is carried out on N^α-
deprotected samples by using the same methods. N-Terminal
solid-phase sequencing runs usually reveal a major portion
10 of the sequence. The remaining sequence may be obtained by
runs of HPLC-fractionated fragments, derived either by
proteolytic cleavage with Asp-N-endoprotease (Boehringer
Mannheim Canada, Laval, Quebec) or by chemical cleavage,
through preferential hydrolysis of the Asp-Pro peptide bond
15 in dilute formic acid.

For the biological assays referred to in the following
examples, human neutrophils isolated from buffy coats of
donor blood were placed in a final suspension of 10^8
cells/ml kept at 0.15 mM MaCl, 0.05 mM $CaCl_2$ at 10°C until
20 use. Competition binding studies were performed according
to the following protocol.   IL-8 was iodinated with
Enzymobead reagent (Bio-Rad) as instructed by the supplier.
Briefly, 1 nmol of IL-8 was mixed with 50 μl of rehydrated
Enzymobead reagent, 50 μl of 0.2 M potassium phosphate, pH
25 7.2, 2 mCl or $Na^{125}I$, and 25 ml of 2% D(+)-glucose and
incubated for 30 min at 21°C. After stopping the reaction
with 50 μl of 1 M KI, the protein is separated from label
by desalting on Bio-Gel P6DG. The ^{125}I-IL-8 preparations
were each analyzed for purity (SDS-polyacrylamide gel
30 electrophoresis) and binding capacity (self-displacement
analysis using freshly isolated human neutrophils) prior to
use. Neutrophils (2 x 10^8) in 120 μl of RPMI 1640 medium
containing 20 mM Hepes, pH 7.3, and 10 mg/ml BSA (binding
medium) are incubated on ice (0-4°C) for 90 min with 1 nM
35 ^{125}I-IL-8 in the presence or absence of cold competitor (10^{-11}
to 10^{-5}). Cells are separated from unbound radioactivity by

-18-

centrifugation for 1 min at 8000 x g through 350 μl of
phosphate-buffered saline (PBS) containing 60 mg/ml BSA
(wash medium) in a Hettich Microliter centrifuge model
2020. The supernatant is aspirated, and the bottom of the
5   tubes containing the cell sediment is sliced off and
counted in a MR 480 automated μ counter (kontron). The K_d
values were determined by calculating nonlinear least
squares fits of the measured data, based on a single
binding site model, and determining the K_d using a LIGAND
10   program as described by Moser, et al (1991) J. Biol. Chem.
266: 10666-10671.

EXAMPLE 1

     Analogs of monomers of IL-8 as described in Table 1
were synthesized according to the preceding methods and
15   folded to produce analog cytokines. In addition, IL-8 1-72
and the 77-residue form of IL-8 were synthesized.

     Cytokine analogs formed from the monomers described in
Table 1 were compared for biological activity according to
in vitro assays described herein. Table 1 shows the
20   ED_{50}values of the indicated analogs estimated from the data
shown in Figures 1, 2, 3, and 4 by determining the protein
concentration (nanomolar) at 50% of the maximum response
that was observed with IL-8 1-72. The binding dissociation
constants (K_d values) were calculated from the competitive
25   binding data shown in Figures 5 and 6 as described above.

Table 1

Analog	Elastase ED$_{50}$	Chemotaxis ED$_{50}$	Binding K$_d$
1-71	12.0	0.38	0.25
77-residue IL-8	25.0	0.69	0.35
3-72	2.5	0.21	0.18
4-72	4.5	0.25	0.22
5-72	1000.0	0.48	0.76
6-72	Undetectable	Undetectable	31.0
7-72	Undetectable	Undetectable	Undetectable
1-72	13.0	0.36	0.25
1-69	7.5	0.14	0.15
1-66	13.0	0.32	0.32
1-63	150.0	1.7	3.4
1-60	220.0	3.9	7.3
1-58	680.0	8.9	22.0
1-54	580.0	6.7	10.0
1-51	680.0	7.1	17.0

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-20-

IL-8 1-72; 77-residue IL-8; 3-72; and, 4-72 analogs demonstrated concentration dependent responses in neutrophil and elastase release and chemotaxis assays. Compared to IL-8 1-72, the 4-72 and 3-72 analogs had

5 respectively, an approximate 3 and 5 fold higher potency in the elastase release assay. The 7-residue IL-8 was approximately 2 fold less potent than IL-8. The 3-72 and 4-72 analogs were both about 2 fold more potent than IL-8 1-72 in the chemotaxis assay. The binding studies show

10 that the 3-72 and 4-72 analogs displaced labelled IL-8 with efficiencies close to that of IL-8 but the 77-residue form required significantly higher concentrations.

The 7-72 and 6-72 analogs were inactive in both the elastase release and the chemotaxis assays. The 5-72

15 analog exhibited an approximately 80 fold lower ED_{50} in the elastase release assay as compared to IL-8 but the chemotaxis potency of the 5-72 analog was only slightly lower. The 5-72 and 6-72 analogs also competed well with IL-8 for neutrophil binding with the 5-72 form showing a

20 stronger ability to compete.

The 1-66 analog had approximately equivalent potency and the 1-69 analog had about a 2 fold higher potency, than the 1-72 form in the elastase release assay. The potency of the 1-63 and 1-60 analogs were respectively,

25 approximately 12 and 17 fold lower than the 1-72 form. Elastase release activity was readily detectable, but about 50 fold lower in potency with the 1-58, 1-54, and 1-51 analogs. The same pattern of relative potencies for the COOH-terminus analogs was observed in the chemotaxis

30 assays.

All the COOH-terminus analogs were capable of competing with IL-8 1-72 for binding to human neutrophils. The 1-72, 1-69, and 1-66 forms were approximately equivalent in competing with the labelled ligand. The 1-63

and 1-60 forms showed an approximate 13 and 29 fold
reduction, respectively. The 1-58, 1-54, and 1-51 forms
showed an approximately 40-80 fold less effective ability
to compete for binding that IL-8 1-72. Thus, the 1-51
5   analog with the entire 21 amino acid COOH-terminal region
deleted had detectable activity and was able to compete
fully for IL-8 binding, although its effectiveness was
reduced.

EXAMPLE 2

10   As shown in Example 1, a COOH-terminally truncated 1-
51 analog showed reduced activity in all three assays as
compared to IL-8 1-72. The elastase release activity assay
was repeated using increasing concentrations of a peptide
synthesized according to the preceding methods having the
15   sequence of IL-8 residues 51-72. The IL-8 51-72 peptide
was employed in increasing concentrations together with the
cytokine analog IL-8 1-51 at 10^{-7} M. Figure 7 shows that
the 51-72 peptide at high concentrations (10^{-4} M) provided
an approximated 2 fold increase in the activity of IL-8 1-
20   51. The 51-72 peptide alone at concentrations up to 10^{-4}
M did not stimulate elastase release, chemotaxis, or
receptor binding.

EXAMPLE 3

        Analog cytokines having residues 5-72; 6-72; and 7-72;
25   respectively, were synthesized according to the method
described herein. Figure 8 shows the results of an assay
for elastase release by human neutrophils pretreated with
cytochalasin B and stimulated with 10^{-8} M IL-8 1-72 in the
presence of increasing concentrations of the analog
30   cytokines IL-8 5-72 (▲); IL-8 6-72 (●); and IL-8 7-72 (■).
The assay was performed as previously described and
elastase release is expressed in relative fluorescence
units (1 unit = 1 pMol 7-amino-4-methylcoumarine produced

-22-

/min). Mean values were determined from duplicate determinations form one out of three similar experiments performed with different neutrophil preparations. Results show that, of the three analogs tested, the 6-72 form was
5   most effective in blocking elastase release activity of IL-8 1-72.

As is shown in Figure 9, samples of 2×10^6 neutrophils were incubated for 90 min at 0-4°C with 10^{-9} M ^{125}I-IL-8 1-72 in the presence of increasing concentrations of unlabelled
10 IL-8 1-72 (o), IL-8 5-72 (◊), IL-8 6-72 (▲) and IL-8 7-72 (□), respectively, and the binding of ^{125}I-IL-8 1-72 was determined. The results are means of duplicate measurements representative of three independent experiments performed according to the methods described
15 herein. The results show a lack of competitive binding by the 7-72 analog. The 5-72 analog demonstrated competitive binding close to that of unlabelled IL-8 1-72. The 6-72 analog demonstrated competitive binding at a reduced level.

EXAMPLE 4

20   The ability of cytokine analog IL-8 6-72 to inhibit neutrophil activation was further investigated. As is shown in Figure 10, release of elastase by cytochalasin B-treated human neutrophils stimulated with 10^{-8} M IL-8 (●) or 10^{-8} M fMet-Leu-Phe (▲) in the presence of increasing
25 concentrations of IL-8 6-72 was investigated. The effect of IL-8 6-72 alone was also tested (o). The results are mean values of duplicates from three experiments with different neutrophil preparations according to the methods described herein. The results show that IL-8 6-72 is
30 ineffective in neutrophil activations and will compete with IL-8 1-72 to block neutrophil activation.

As is shown in Figure 11, migrations of human

neutrophils as the indicated concentrations of IL-8 6-72 in
the presence (●) and absence (○) of 10^{-8} M IL-8 1-72 was
investigated. The results are relative values with respect
to non-inhibited controls and are mean values of duplicates

5   form two independent experiments with different neutrophil
preparations performed according to the procedures
described herein. The results show that, in respect of
neutrophil chemotaxis, IL-8 6-72 has a much decreased
activity as compared to IL-8 1-72 and, the former will

10   compete with the latter to inhibit chemotaxis.

The formation of H_2O_2 by human neutrophils after
stimulation with 10^{-8} M IL-8 1-72 in the presence of IL-8 6-
72 at concentrations varying form $0-10^{-6}$ M was tested.
Increasing concentrations of IL-8 6-72 resulted in a

15   reduction of the amount to $H_2 O_2$ produced. IL-8 6-72 alone
was ineffective in stimulating the respiratory burst.

EXAMPLE 5

As is shown in Figure 12, IL-8 6-72 inhibits
neutrophil activation by IL-8 1-72 (●), GROα (■) and NAP-2

20   (▲). The figure shows elastase release by cytochalasin B-
treated human neutrophils stimulated with IL-8 1-72 and the
homologues at 10^{-8} M in the presence of increasing
concentrations of IL-8 6-72. The results are mean values
of duplicate determinations from one out of two similar

25   experiments with different neutrophil preparations,
performed according to the procedures described herein.
NAP-2 refers to neutrophil activating peptide-2 as
described by Walz and Baggiolini (1989) Biochem. Biophys.
Res. Commun. 159: 969-975. GROα refers to a substance

30   having melanoma growth stimulating activity described in
Richmond, et al (1988) Embo. J. 7: 2025-2033.

The behaviour of the 5-72 analog is that of a partial
antagonist wherein receptor binding is relatively high but

-24-

effectiveness in receptor signalling is reduced. Given its binding affinity, it can be expected that cytokine analog IL-8 5-72 will be an effective IL-8 mediating agent, particularly in circumstances where its chemotaxis activity is desirable.

EXAMPLE 6

The effect of amino acid replacement on the activity of interleukin-8 analogues was also explored, using compounds prepared by t-boc-based solid phase peptide synthesis. Results are presented in table 2 (Orn = ornithine).

Table 2

Analogue/Variant	1	2	3	4	5	6	7	8	9	Kd(nM)	IC_{50}(µM)
			N-terminal sequence								
IL-8(1-72)	Ser	Ala	Lys	Glu	Leu	Arg	Cys	Gln	Cys...	0.25	
IL-8(4-72)				Glu	Leu	Arg	Cys	Gln	Cys...	0.22	
IL-8,ELQ(7-72)				Glu	Leu	Gln	Cys	Gln	Cys...	650	1.1
IL-8,ELL(7-72)				Glu	Leu	Leu	Cys	Gln	Cys...	1290	0.7
IL-8,ELK(7-72)				Glu	Leu	Lys	Cys	Gln	Cys...	220	1.8
IL-8,ELOrn(7-72)				Glu	Leu	Orn	Cys	Gln	Cys...	400	5.4
IL-8,ELNle(7-72)				Glu	Leu	Nle	Cys	Gln	Cys...	170	1.0
IL-8,AAR(7-72)				Ala	Ala	Arg	Cys	Gln	Cys...	7.9	0.3
IL-8(5-72)					Leu	Arg	Cys	Gln	Cys...	0.8	
IL-8,IR(7-72)					Ile	Arg	Cys	Gln	Cys...	16	0.3
IL-8,QR(7-72)					Gln	Arg	Cys	Gln	Cys...	34	2.1
IL-8(6-72)						Arg	Cys	Gln	Cys...	50	0.3
IL-8(7-72)							Cys	Gln	Cys...	>10,000	

From these results, it is evident that all analogues extended N-terminally from the core IL-8(7-72) region exhibit neutrophil binding affinity (Kd) that is at least in the μM range. Particularly useful as antagonists of interleukin-8 activity are those compounds having both a Kd value that is less than about 50 nM and an IC_{50} value that is less than about 2.5μM, such as IL-8,AAR(7-72); IL-8,IR(7-72); IL-8,QR(7-72) and IL-8(6-72). Remarkably, these results also reveal a second class of IL-8 analogs capable of antagonizing the action of IL-8; those that despite having Kd values exceeding about 50nM, nevertheless inhibit IL-8-mediated elastase release at concentrations not exceeding about 2.5uM. Such potent IL-8 analogs include IL-8,ELL(7-72); IL-8,ELK(7-72); and IL-8,ELNle(7-72).

EXAMPLE 7 - In vivo studies

The effect of various interleukin 8 analogs on inflammation was evaluated in vivo using the rabbit plasma exudation dermal assay reported previously by Beaubien et al, in Biochem. J., 1990, 271:801. In this model, inflammatory activity in the peritoneal exudate is monitored by its ability to induce oedema formation and neutrophil accumulation in rabbit skin. Briefly, rabbits were anaesthetized with sodium pentobarbitone, the dorsal skin was shaved and radiolabelled tracers (^{125}I-albumin and, in some experiments, ^{111}In-neutrophils) were injected intravenously. Test samples suspended in phosphate buffered saline were injected intradermally (n=6). After four hours, the animals were killed with an anaesthetic overdose. The dorsal skin was removed, and the injection sites were punched out and counted for radioactivity in a multi-well Cobra Auto-Gamma radiation counter with spill-over correction (Packard).

Results (mean $\pm$ S.E.M.), presented in Figures 14 and 15, are expressed as μl of plasma/skin site (oedema

formation) and ^{111}In-neutrophil accumulation/skin site. It
will be noted that both IL-8(6-72) and the IL-8,AAR(5-72)
analogs exhibited antagonism of IL-8(1-72)-mediated oedema
formation, and that neither analog exhibited a significant
5 chemotactic effect on neutrophil accumulation.

Various changes and modifications may be made in
practising this invention without departing from the spirit
and scope thereof.

-28-

WE CLAIM:

1. An interleukin-8 analog, the analog comprising an amino acid sequence substantially equivalent to the human IL-8 1-72 sequence beginning at residue 4 and continuing C-
5   terminally at least to residue 51, wherein at least one of residues Glu^4, Leu^5 and Arg^6 is replaced or deleted.

2. An interleukin-8 analog, as defined in claim 1, wherein the amino acid sequence thereof continues C-terminally at least to residue 66.

10 3. An interleukin-8 analog, as defined in claim 1, wherein the amino acid sequence thereof continues C-terminally at least to residue 69.

4. An interleukin-8 analog, as defined in claim 1, wherein the amino acid sequence thereof continues C-terminally to
15 residue 72.

5. An interleukin-8 analog, as defined in any one of claims 1-4, wherein residue Arg^6 is replaced.

6. An interleukin-8 analog, as defined in any one of claims 1-5, wherein residue Leu^5 is replaced.

20 7. An interleukin-8 analog, as defined in claim 5, wherein residue Arg^6 is replaced by an amino acid selected to confer on said analog a Kd value greater than about 50nM and an IC$_{50}$ value, as determined by the elastase release assay, of less than about 2.5μM.

25 8. An interleukin-8 analog, as defined in claim 5, wherein residue Arg^6 is replaced by an amino acid selected from leucine, norleucine and lysine.

SUBSTITUTE SHEET

-29-

9. An interleukin-8 analog, as defined in claim 8, which is an analog of human IL-8(4-72).

10. An interleukin-8 analog, as defined in any one of claims 1-4, which is human IL-8,Ala^{4}Ala^5(6-72).

5   11. An interleukin-8 analog, as defined in any one of claims 1-4, wherein residue Glu^4 is deleted.

12. An interleukin-8 analog, as defined in claim 11, wherein residue Leu^5 is replaced.

13. An interleukin-8 analog, as defined in claim 12,
10 wherein residue Leu^5 is replaced by an amino acid selected such that the analog retains a Kd value that is greater than about 100nM and an IC$_{50}$ value, as determined in the elastase release assay, of less than about 2.5μM.

14. An interleukin-8 analog, as defined in claim 13,
15 wherein residue Leu^5 is replaced by an amino acid selected from glutamine and isoleucine.

15. An interleukin-8 analog, as defined in claim 14, which is human IL-8,Ile^5(6-72).

16. An interleukin-8 analog, as defined in claim 14, which
20 is human IL-8,Gln^5(6-72).

17. An interleukin-8 analog, as defined in claim 11, which is human IL-8(5-72).

18. An interleukin-8 analog, as defined in claim 11, wherein residue Leu^5 is deleted.

25 19. An interleukin-8 analog, as defined in claim 18, which is human IL-8(6-72).

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20.   A pharmaceutical composition comprising an interleukin-8 analog as defined in any preceding claim, and a suitable carrier therefor.

21. A biologically active analog of human interleukin-8, the analog having an amino acid sequence substantially equivalent to the IL-8 sequence beginning at residue 4 and continuing C-terminally to a residue between residues 50 and 72.

22. A biologically active human interleukin-8 analog as defined in claim 21, the analog having a sequence that continues C-terminally at least to residue 66.

23. A biologically active human interleukin-8 analog as defined in claim 22, which is IL-8[3-69].

24. A pharmaceutical composition comprising a biologically active human interleukin-8 analog as defined in any one of claims 21-23, and a suitable carrier therefor.

25. The use of a biologically active, human interleukin-8 analog as defined in any one of claims 21-23 in the preparation of a neutrophil activating medicament.

26. The use of a neutrophil-binding analog of human interleukin-8 as defined in any one of claims 1-19 in the preparation of a medicament for controlling inflammation.

SUBSTITUTE SHEET.

1/13

FIG. 1.
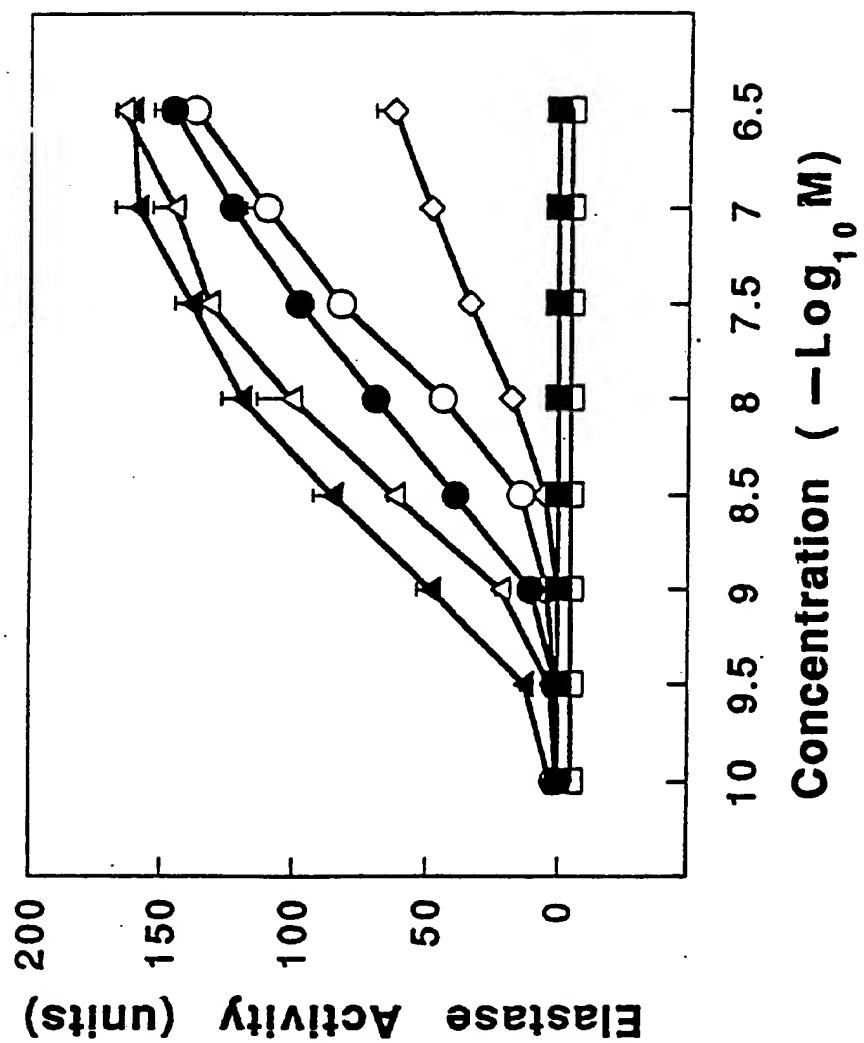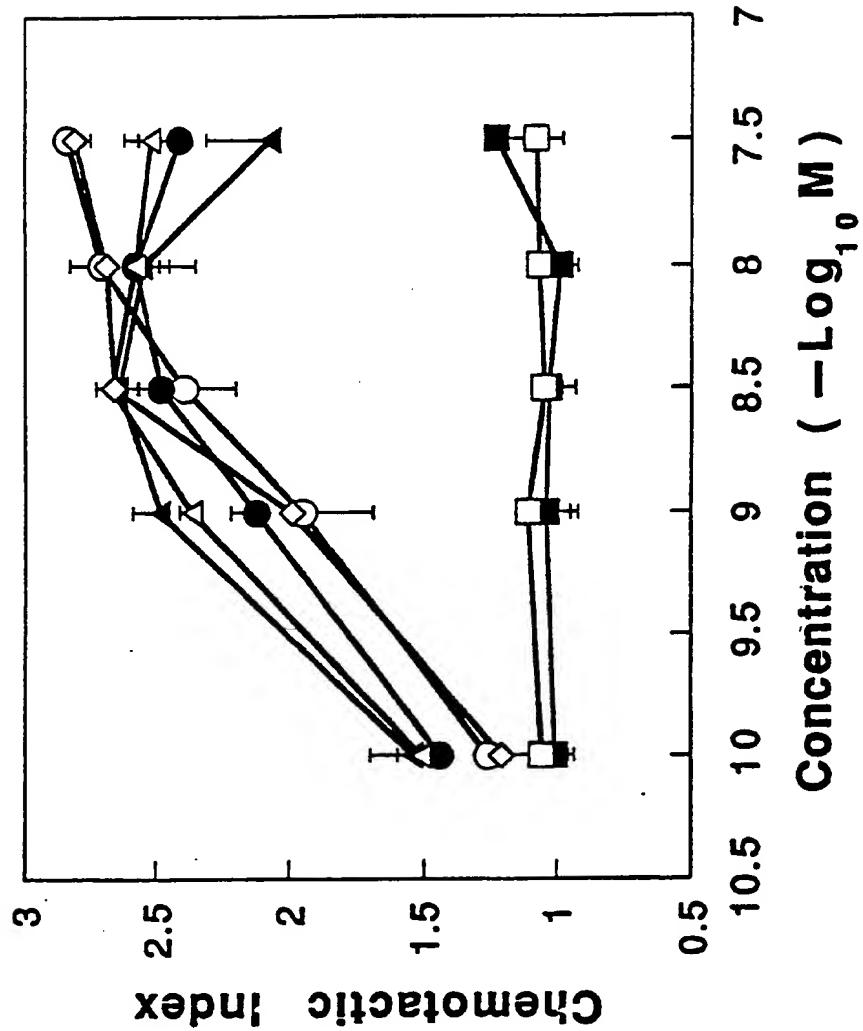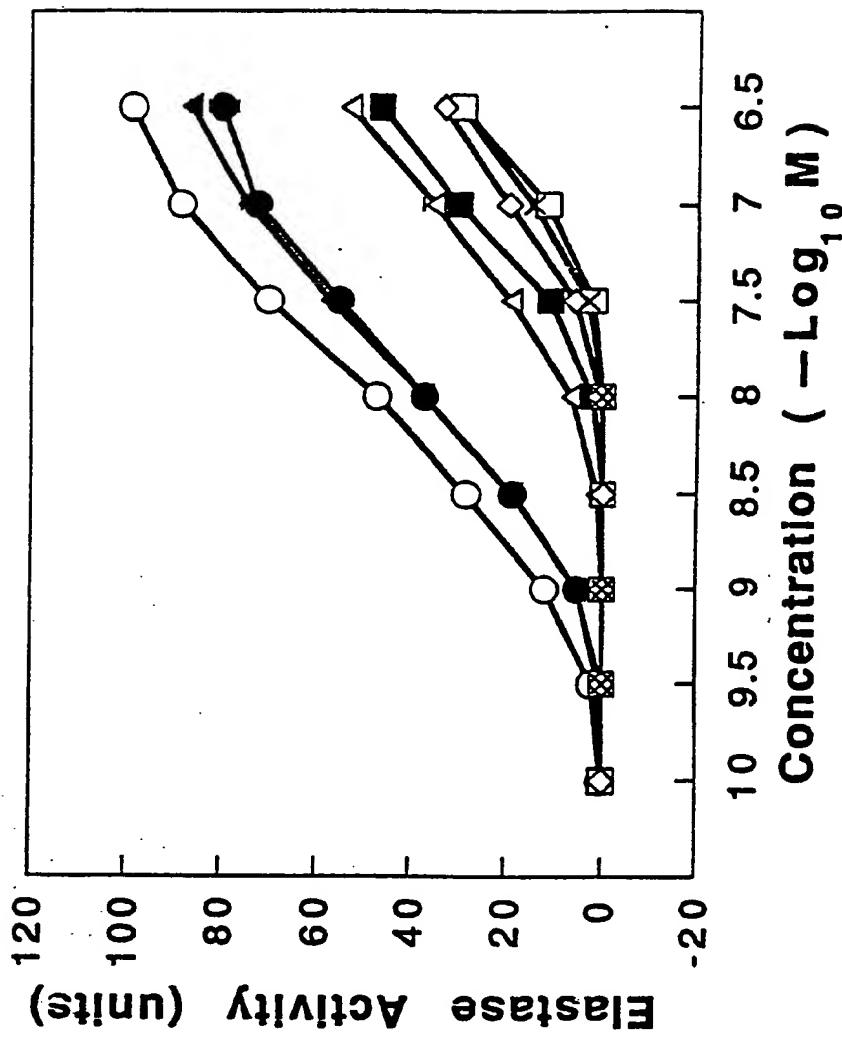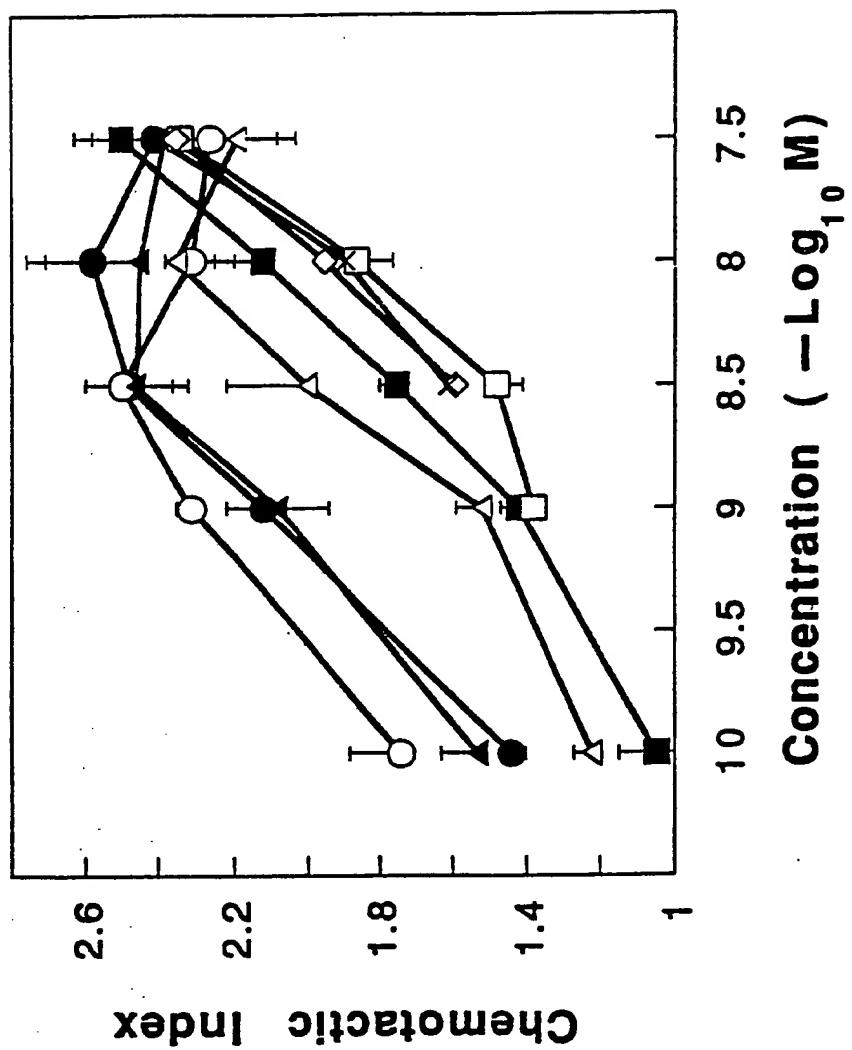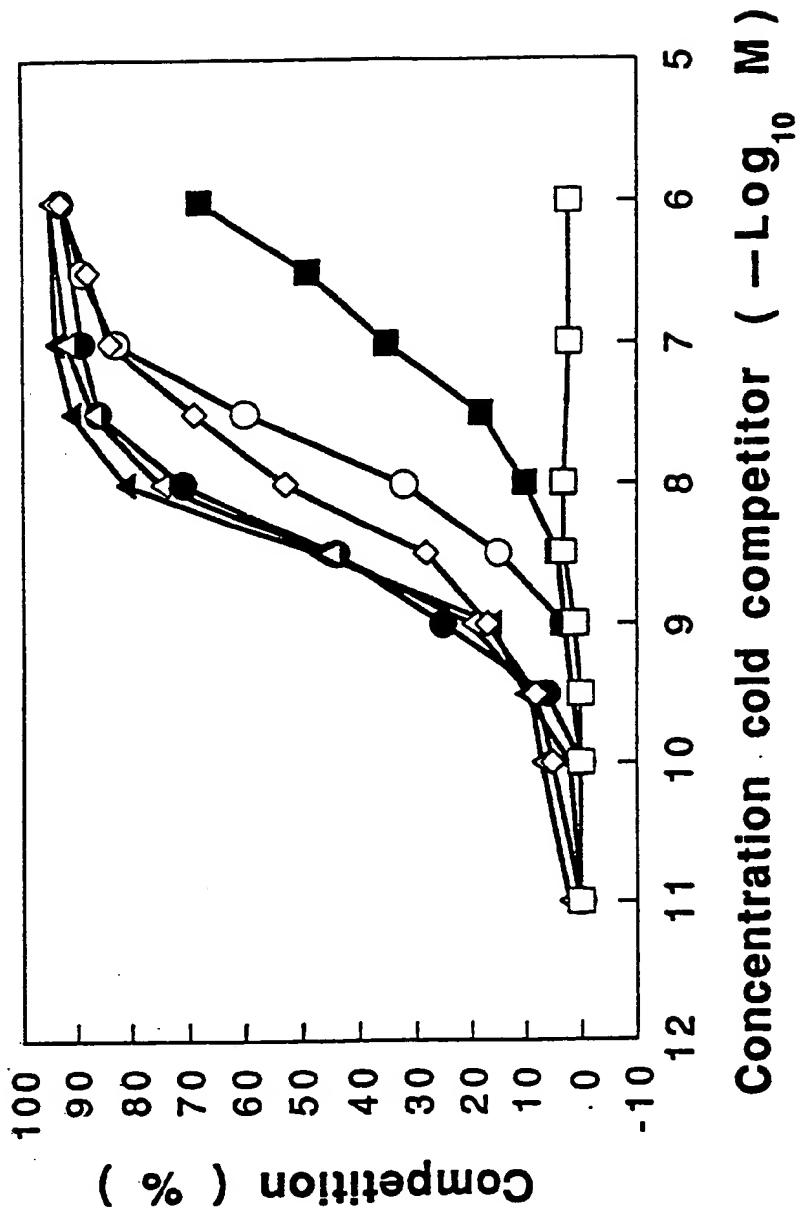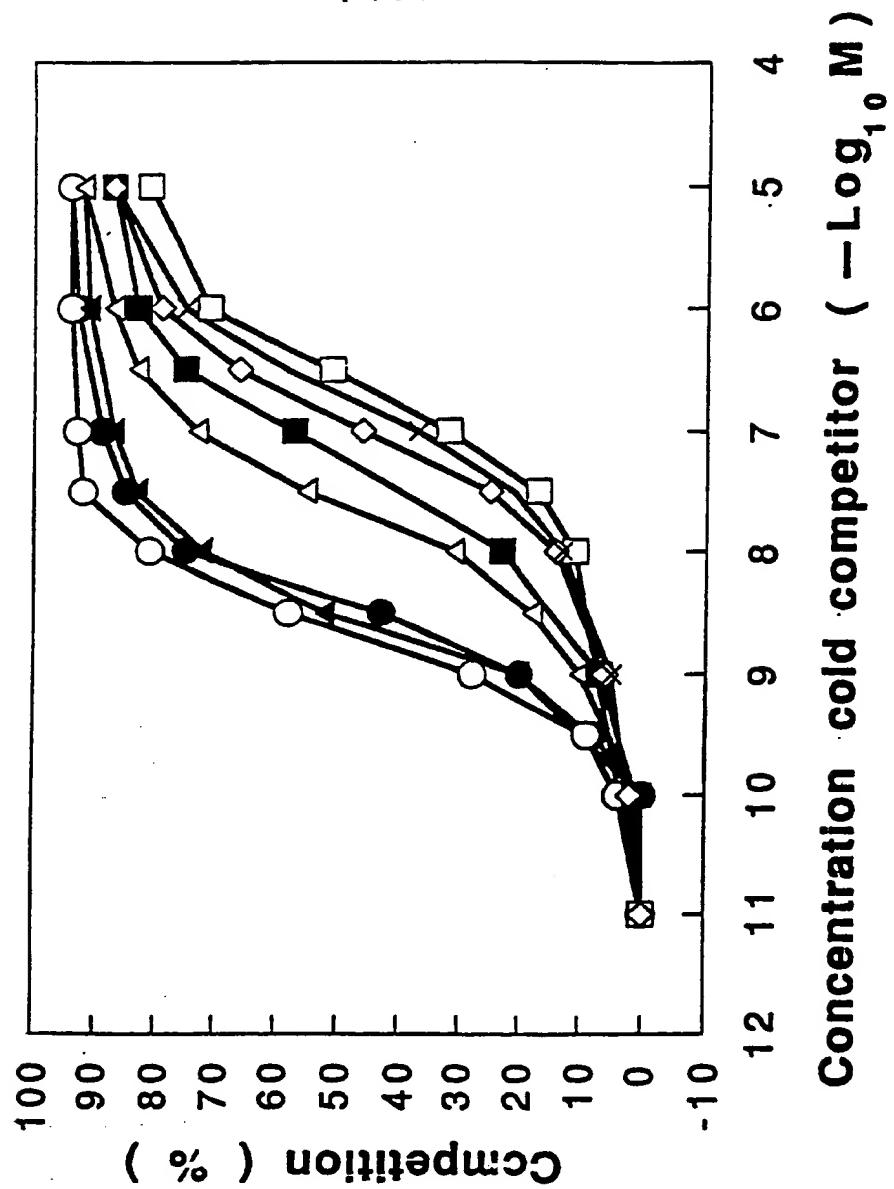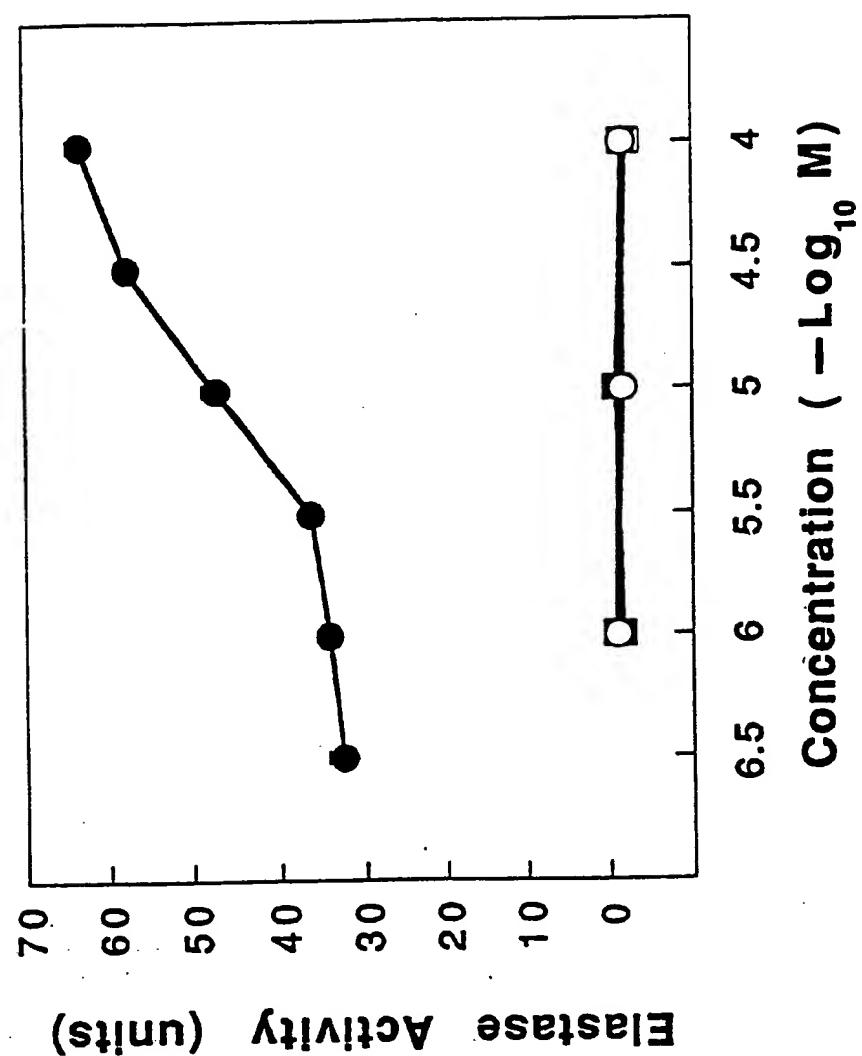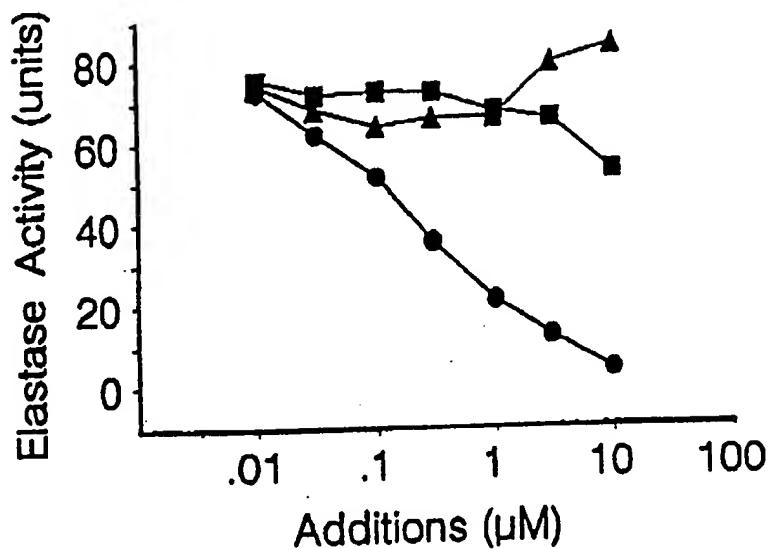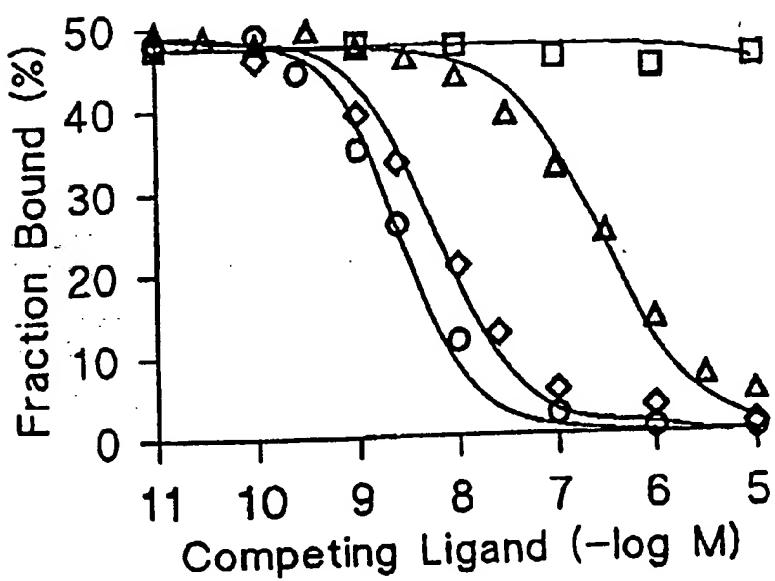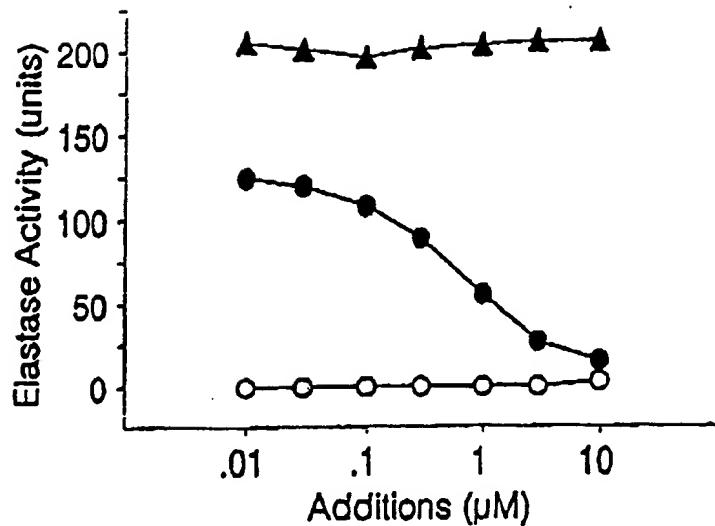
FIG. 2.

3/13

FIG. 3.

FIG. 4.

4/13

5/13

FIG.5.

FIG.6.

6/13

7/13

FIG.7.

FIG.8.

FIG.9.

FIG.10.


FIG.11.

FIG. 12.

11/13
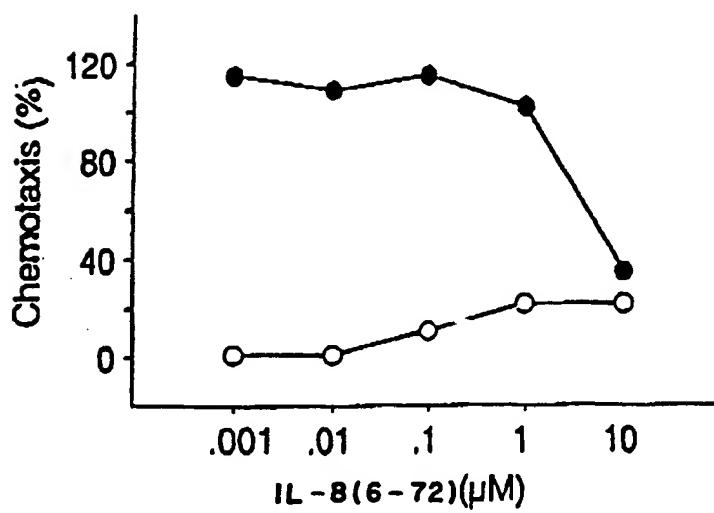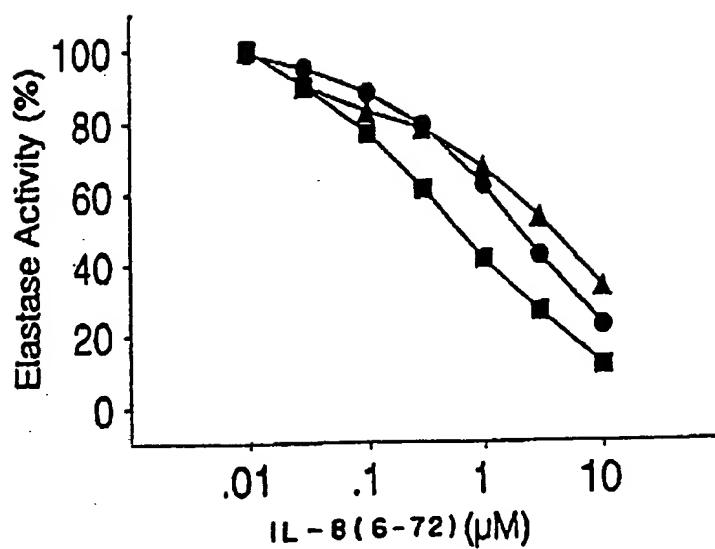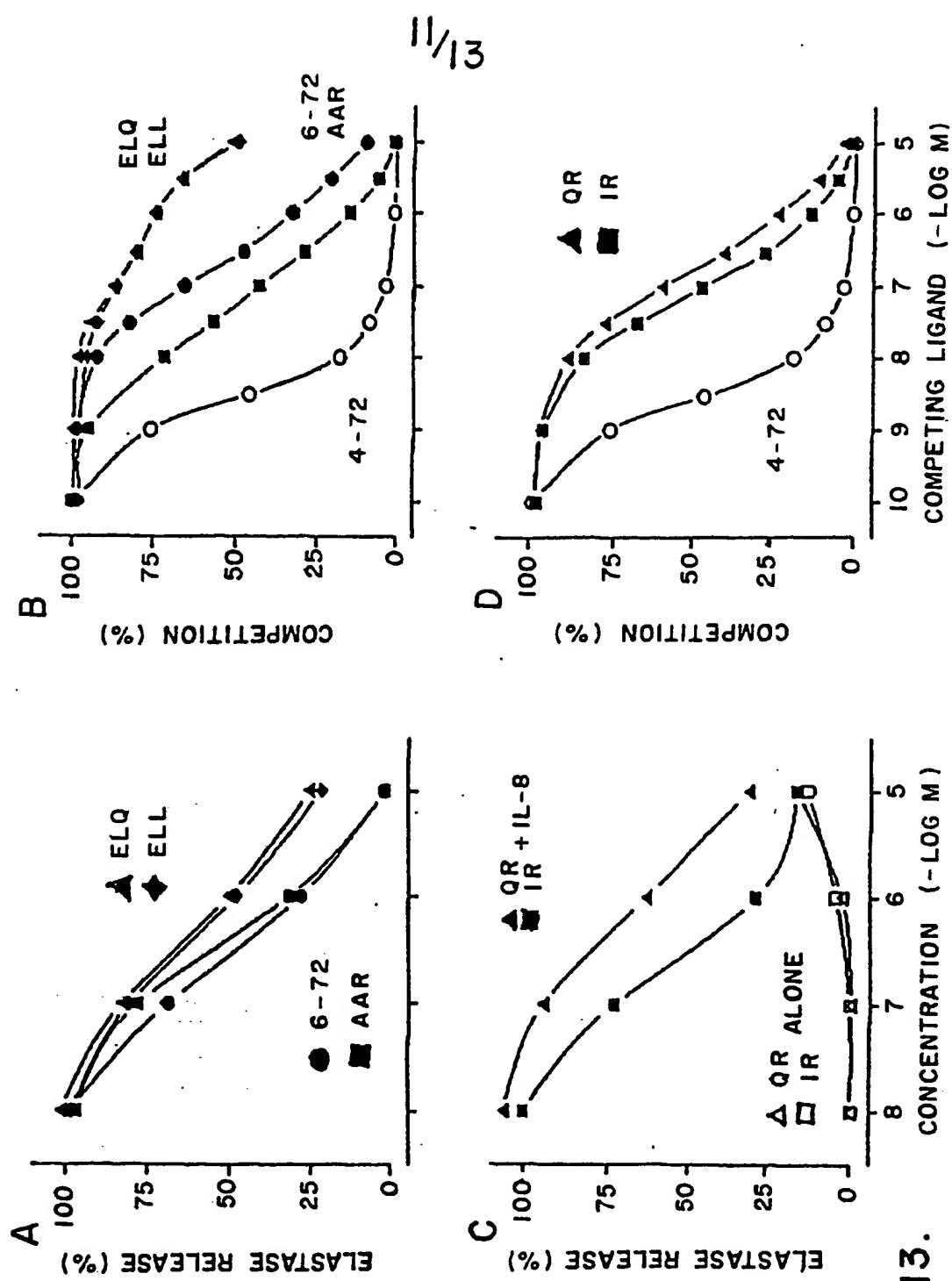
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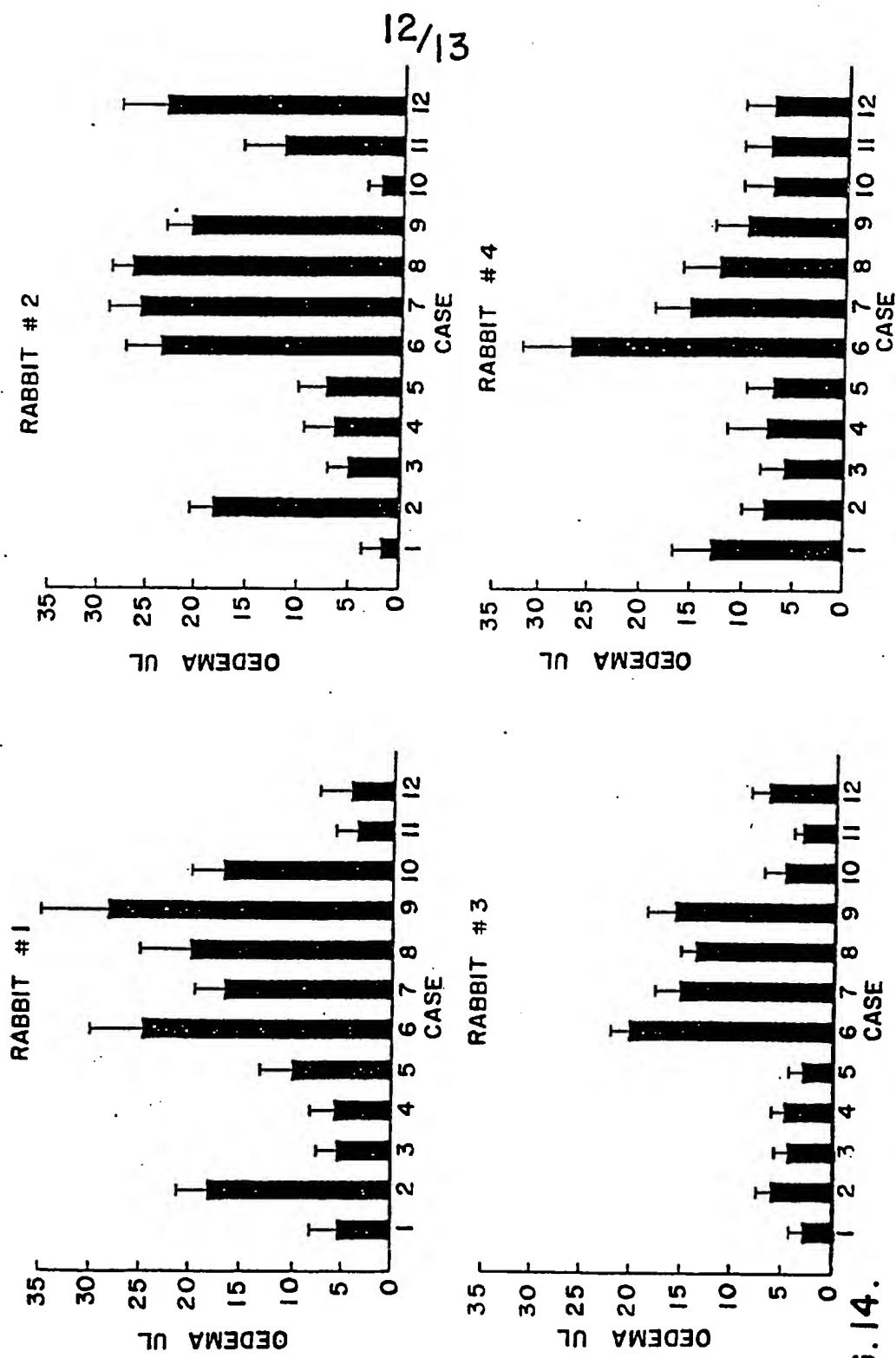
FIG. 14.
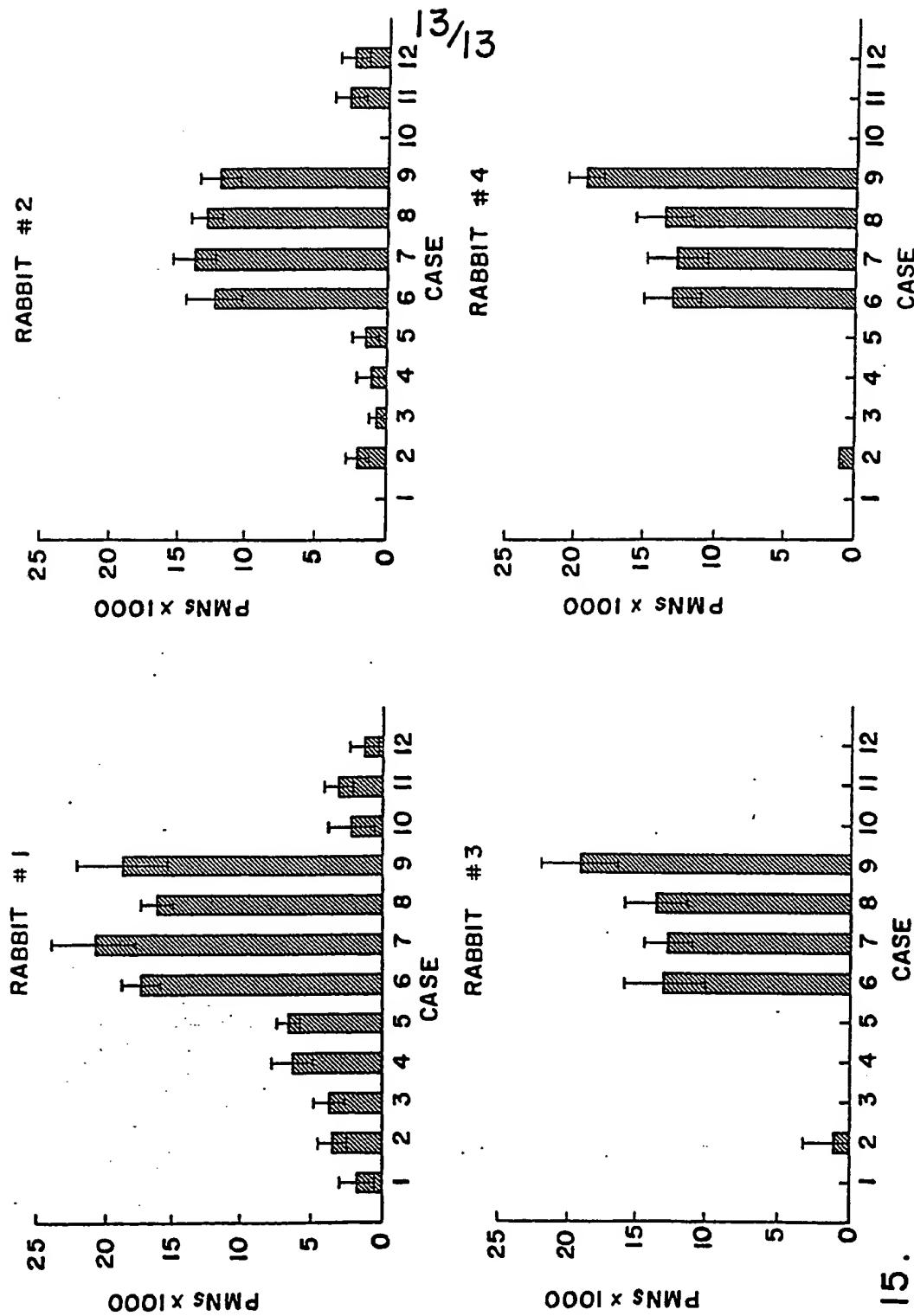
FIG. 15.

I. CLASSIFICATION OF SUBJECT MATTER   (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K13/00;   C12P21/02;   A61K37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12P ; C12N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[o]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 266, no. 34, 5 December 1991, BALTIMORE US pages 23128 - 23134 IAN CLARK-LEWIS ET AL 'Structure -activity relationships of interleukin-8 determined using chemically synthesized analogs' see the whole document ---	1,4
A	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 266, no. 28, 5 October 1991, BALTIMORE US pages 18989 - 18994 C. A. HEBERT ET AL 'Scanning mutagenesis of Interleukin-8 identifies a cluster of residues required for receptor binding' see the whole document --- -/--	1

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not
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"L" document which may throw doubts on priority claim(s) or
which is cited to establish the publication date of another
citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or
other means

"P" document published prior to the international filing date but
later than the priority date claimed

"T" later document published after the international filing date
or priority date and not in conflict with the application but
cited to understand the principle or theory underlying the
invention

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cannot be considered novel or cannot be considered to
involve an inventive step

"Y" document of particular relevance; the claimed invention
cannot be considered to involve an inventive step when the
document is combined with one or more other such docu-
ments, such combination being obvious to a person skilled
in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 FEBRUARY 1993	05.03.93

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	LE CORNEC N.D.R.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	WO,A,8 904 836 (SANDOZ AG) 1 June 1989 see page 8, line 20 - page 9, line 8; claim 6 -----	21-22, 24-26

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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